

1 **Genetic diversity of *Burkholderia contaminans* isolates from cystic**
2 **fibrosis patients in Argentina**

3
4 **Pablo Martina¹, Marisa Bettiol², Cecilia Vescina², Patricia Montanaro³, M.**
5 **Constanza Mannino¹, Claudia I. Prieto¹, Carlos Vay⁴, Dieter Naumann⁵, Juergen**
6 **Schmitt⁶, Osvaldo Yantorno¹, Antonio Lagares⁷ and Alejandra Bosch^{1*}**

7
8 ¹Centro de Investigación y Desarrollo de Fermentaciones Industriales (CINDEFI-
9 CONICET, CCT-La Plata), Facultad de Ciencias Exactas, Universidad Nacional de La
10 Plata, Calle 50 N° 227 entre 115 y 116, (1900) La Plata, Argentina

11 ²Sala de Microbiología, Hospital de Niños de La Plata “Sor María Ludovica”. La Plata,
12 Argentina

13 ³Servicio de Bacteriología, Hospital Santísima Trinidad de Córdoba, Córdoba, Argentina

14 ⁴Servicio de Bacteriología, Hospital de Clínicas, Ciudad Autónoma de Buenos Aires,
15 Argentina

16 ⁵Robert Koch Institute, P34, Nordufer 20, 13353 Berlin, Germany

17 ⁶Synthon GmbH, Im Neuenheimer Feld 583, 69120 Heidelberg, Germany

18 ⁷Instituto de Biotecnología y Biología Molecular (IBBM-CONICET, CCT-La Plata),
19 Facultad de Ciencias Exactas, UNLP, La Plata, Argentina

20
21 **Running Title:** *B. contaminans* in cystic fibrosis

22
23 **Key words:** *Burkholderia contaminans*, BOX-PCR fingerprinting, chronic infection

24
25 *E-mail address: bosch@quimica.unlp.edu.

26
27 Tel/Fax: +54 221 4833794 int 122

28

29

30 **ABSTRACT**

31 One hundred twenty *Burkholderia cepacia* complex isolates collected during 2004–2010
32 from 66 patients in two cystic fibrosis reference centers in Argentina were analyzed. *B.*
33 *contaminans* was the species most frequently recovered (57.6%) followed by *B.*
34 *cenocepacia* (15%), a species distribution not reported so far. The *recA*-PCR-based
35 techniques applied to the *B. contaminans* isolates revealed that 85% of the population
36 carried the *recA*-ST-71 allele. Our results showed the utility of BOX-PCR genotyping in
37 analyzing *B. contaminans* diversity. This approach allowed us to address a clonal
38 transmission during an outbreak and the genetic changes occurring in infecting bacteria
39 over the course of chronic infection.

40

41
42 *Burkholderia cepacia* complex species are capable of causing chronic and often severe
43 respiratory-tract infections in cystic fibrosis (CF) patients and other types of infections in
44 immunocompromised patients. Although many CF patients remain infected by these
45 bacteria and relatively healthy for prolonged periods, others either have a severe decline in
46 their pulmonary status, or die shortly after the initial colonization (2). In Argentina, the
47 first reports of *B. cepacia* complex bacteria infecting CF patients started around 1990. At
48 that time *Burkholderia* spp. were recovered sporadically and with a very low prevalence
49 (<0.1%). The prevalence started to increase over the last decade from 0.2 to 3.6%
50 depending on the medical center. In early 2004 an outbreak involving main Argentine CF-
51 care centers occurred, and the proportion of patients from whom *B. cepacia* complex
52 species could be recovered ranged from 19 to 36% (5, 18, 22). Currently, through strict
53 infection-control procedures, the prevalence of *Burkholderia* spp. in local patients has
54 decreased to approximately 10%. Recent worldwide surveillance studies concerning the
55 distribution of *B. cepacia* complex species in CF patients have cited as the most frequently
56 recovered species *B. multivorans* and *B. cenocepacia*. These species account for
57 approximately 80% of the infected patients, and the prevalence between them is
58 geographically and temporally dependent (13, 15, 19). Unlike this worldwide situation, in
59 Argentina a relatively high prevalence of *B. contaminans* followed by *B. cenocepacia* has
60 been encountered (18, 22). To our knowledge, no other geographical region has been
61 reported in the literature with such a high occurrence of *B. contaminans* infecting CF
62 patients. In view of this particular scenario coupled with the lack of biodiversity
63 information available for *B. contaminans* species, we decided to study this local
64 population by *recA*-PCR-based techniques and by repetitive element sequence-based PCR
65 (rep-PCR) approaches. These methods allowed us to address the genetic diversity of *B.*

66 *contaminans* isolates recovered during that outbreak along with the genetic changes
67 occurring in the infecting bacteria over the course of a chronic infection.

68 **Distribution of *Burkholderia cepacia* complex species in CF patients.** This study
69 involved the analysis of a total of 120 clinical *Burkholderia cepacia* complex isolates
70 recovered from 66 CF patients from 2004 through 2010 and 13 reference strains (Table
71 S1, Supplementary Material). The isolates were recovered from sputum samples of CF
72 patients treated at 2 main CF reference centers of Argentina (Hospital de Niños Sor María
73 Ludovica, La Plata [HNLP], and Hospital Santísima Trinidad, Córdoba [HST], located
74 800 km from La Plata to the north; plus one hospital (Hospital Dr. Pedro Moguillanski,
75 Cipoletti, Río Negro [HNRN], 1,200 km from La Plata to the south. Twenty-two isolates
76 (indicated with a “v” in Table S1, Supplementary Material) were withdrawn from patients
77 infected during the *B. cepacia* complex outbreak, and a cohort of 51 isolates was
78 recovered from 21 patients chronically infected with *B. cepacia* complex bacteria. Patients
79 were considered chronically infected when three positive cultures of *B. cepacia* complex
80 strains were isolated within a 6-month period (24). Bacterial isolation was performed on
81 *B. cepacia* selective agar medium (BCSA, Lab. Britania S. A., Argentina) according to
82 clinical microbiology practice recommendations for respiratory-tract specimens from CF
83 patients (9, 16) and were phenotypically characterized by means of conventional
84 biochemical techniques (9, 16, 23, 33). The species-level identification was achieved by
85 *recA*-PCR-based techniques. The *recA* gene (1040 bp) was amplified through the use of
86 BCR1 and BCR2 primers (21) and the amplified products subjected to restriction-
87 fragment-length-polymorphism (RFLP) analysis with the restriction enzyme *HaeIII*
88 (Promega, Inc.; 21). The restriction-PCR patterns obtained were analyzed and compared
89 with those reported in the literature (11, 12, 21, 29). The amplified products were purified
90 through the use of the QIAquick PCR-purification kit (Qiagen Inc., CA, USA), and
91 sequenced on both strands with the BigDye Terminator Cycle Sequencing Ready Reaction

92 kit (Applied Biosystem, Foster City, CA, USA) at MacroGen, Inc. (Seoul, Korea). A final
 93 identification was achieved by *recA*-gene-DNA-sequence analysis and *gyrB* DNA
 94 fragment (1,900 bp) sequencing when an identification remained ambiguous. The *recA*-
 95 sequence type (*recA*-ST) within each species was obtained by comparing the sequence of
 96 a 398 bp *recA* fragment against the Multi Locus Sequence Typing (MLST) database
 97 (<http://pubmlst.org/bcc/>) (2).

98 The analysis of species distribution among the patients studied showed that 8 out of the
 99 17 species described for the *B. cepacia* complex were infecting the local CF population,
 100 with a remarkably high representation of *B. contaminans* that accounted for 57.6% of the
 101 infected patients. The data on the species of the first isolate available from each patient
 102 indicated that *B. cenocepacia* was the second most frequent species, it encountered at a
 103 frequency of almost 15% of the patients, followed by *B. cepacia* at 7.5%, *B. multivorans*
 104 at 6.0%, *B. stabilis* and *B. vietnamiensis* at 5%, *B. seminalis* at 3.0 %, and *B. ambifaria* at
 105 1.5%.

106 In order to examine the genetic diversity among the *B. contaminans* isolates, sequence
 107 analysis of a 900 bp fragment of the *recA* gene was performed over the 79 *B. contaminans*
 108 isolates (Table S1, Supplementary Material). The phylogenetic relationship among them
 109 was investigated through the use of the MEGA version 4.0 software
 110 (<http://www.megasoftware.net>; Fig. 1). Isolates were clearly separated into one large
 111 homogenous cluster that grouped 85% of the *B. contaminans* population under study along
 112 with four small clusters containing only 5 isolates. When the sequence of the 398 bp DNA
 113 fragment from the *recA* gene was compared against the MLST database (2), all the
 114 bacteria included in the main cluster showed to belong to the *recA*-ST-71 allele; while 4 of
 115 the isolates grouping outside this cluster displayed different *recA*-ST alleles (ST-64, -166,
 116 and -243). The isolates grouped in the main cluster had a *recA*-*Hae*III-restriction pattern
 117 corresponding to the K-type, whereas the isolates outside this group gave profiles of K,

AT, and J (Table S1, Supplementary Material). Interestingly, one of the isolates (HNLP 1001) exhibited the J *recA*-RFLP pattern which was reported to be representative of the species *B. cenocepacia*, *B. stabilis*, *B. lata*, *B. seminalis*, and *B. arboris* (21,27, 28), but so far not documented for *B. contaminans*. It is notable that none of the *recA* sequences corresponding to isolates that grouped in the main cluster was represented by the *recA* reference sequences available at GenBank. It should also be remarked that two reference sequences displaying the *recA*-ST-71 allele, those belonging to two isolates recovered from CF patients in Brazil (*B. contaminans* R18428 and R9929), clustered at a short distance from the homogenous cluster (Fig. 1). A further exploration of a possible regional incidence of *B. contaminans* among isolates circulating in Brazil should prove interesting. In this regard, the incidence of RFLP K pattern strains in CF patients seen at two different hospitals in Brazil (1, 12) might also be indicating the existence of a regional spreading of *B. contaminans* between bordering countries.

Genetic diversity among *B. contaminans* isolates with the *recA*-ST-71 allele as investigated by Rep-PCR DNA fingerprinting analysis. The low level of polymorphism in the *recA* gene encountered in the *B. contaminans* population studied here prompted us to explore the genetic diversity among the isolates carrying the *recA*-ST-71 allele by repetitive element sequence-based PCR (rep-PCR; 30). This subset of 75 *B. contaminans* isolates was analyzed by REP-, ERIC- and BOX-PCRs. Amplification reactions were performed in a My Cycler Thermal Cycler (BIO-RAD, USA) with the REP1R-I, REP2-I, ERIC1R-I, and BOX-A1R primers (30). The amplicons generated were electrophoresed in agarose gels and the banding patterns obtained analyzed by means of the GelCompar II version 2.1 software package (Applied Maths, Kortrijk, Belgium). The profiles yielding a band similarity of 85% or greater were considered related clones for all assays. According to this criterion, both REP- and ERIC-banding patterns displayed a very limited diversity. With the REP primers, the isolates showed only four different banding patterns, with one

144 being observed in 92% of the isolates. Similarly, when ERIC-PCR primers were used, 5
145 different patterns were found, with one subtype including 89% of the isolates (data not
146 shown). The highest discrimination power was obtained when BOX-PCR was performed.
147 This fingerprint typing yielded 10 distinct amplification profiles (B1 to B10; Fig. 2). The
148 distribution of the isolates' amplification profiles was as follows: the B8 subtype, 50%; the
149 B1 subtype, 22.9%; the B6 and B7 subtypes, 6.75%; the B9 subtype, 5.4%; and the B2,
150 B3, B7, B5, and B10 subtypes, 1.35%, these comprising only 1 isolate each.

151 Through the use of the first *B. contaminans* isolate recovered from each patient over the
152 period 2004–2010, the incidence of each BOX subtype was determined. Three subtypes,
153 B1, B7, and B8 were the most abundant patterns found, all together accounting for 75% of
154 the isolates. The B6 subtype was observed in 3 patients, while the remaining 6 subtypes
155 were unique fingerprints observed in only one patient each (Fig. 3A). With respect to the
156 geographical distribution, whereas the isolates with B7 and B8 subtypes were found at
157 both references centers at a remarkably high incidence, all the other BOX subtypes were
158 present at only one of the two locations (Fig. 3B). Thus, although the interpatient
159 transmission of *B. contaminans* with the B7 and B8 BOX subtypes could very likely have
160 been a dominant occurrence at both CF centers, other sources of new infections were
161 clearly present. Regarding the prevalence of each BOX subtype over time, during the
162 period of the outbreak (2004–2005), the CF patients were in most instances infected with
163 the B8 (55%), B7 (15%), and B6 (11%) subtypes—but also in a much lower proportion
164 with isolates belonging to other genotypes (B2, B3, B4, B9, and B10; Fig. 3C). We
165 therefore infer that the isolates belonging to the B8 genotype (and to a lesser extent to B7),
166 were efficiently transmitted at the outbreak period to produce a clonal spreading among
167 the patients (Fig. 3B and 3C). Nevertheless, that other different unique BOX subtypes (*e.*
168 *g.*, the B2, B3, B4, B9, and B10 subtypes) were also present should be stressed (Fig. 3C).
169 Hence, as previously reported for different *Burkholderia* spp. outbreaks (4), although a

high number of infections could have been the result of particular BOX subtype patient transmission, other sources of infection—such as through the environment or industrial products—might have accounted for the CF infections within that same time period. Additionally, it should be noted that since 2005 onward, isolates belonging to BOX subtypes B1 and B8 most notably seem to be responsible for new infections in CF patients. The PCR-fingerprinting techniques have been successfully applied previously for *B. cenocepacia*, *B. cepacia*, *B. multivorans*, *B. dolosa*, and *B. pseudomallei* clinical-isolate discrimination (4, 8). In fact, the application of ERIC- and BOX-PCR have proven to be valid technical alternatives to pulsed-field gel electrophoresis (6, 8, 10). In the present work, we were able to demonstrate that BOX-PCR is also a reliable typing method to examine the genetic relatedness among *B. contaminans* isolates so as to enable the identification of a spreading of two different genotypes within a very closely related group of isolates.

***B. contaminans* diversity in chronically infected patients.** CF patients are known to be susceptible to respiratory-tract chronic infection by certain *Burkholderia* species (3). Both the persistence of the same strain and the colonization with a different strain during the course of long-term infection have been reported (3, 7, 20). In 8 out of the 21 chronically infected patients analyzed in this work during the 7-year period, more than one *Burkholderia* species was recovered during the course of the infection (Table 1, rows 1 to 3), either as a consequence of coinfection or through subsequent colonization by different species (so-called “species replacement”). In contrast, in the remaining 13 chronically infected patients only one species (*B. contaminans*) was obtained (Table 1, rows 4 and 5). Upon consideration of the BOX subtypes of serially recovered isolates during the infection of each of these 13 patients we found that 5 patients remained infected with isolates carrying the same BOX subtype, whereas the other 8 evidenced a change in their isolate’s BOX subtype during chronic infection. The latter result again could have occurred either

196 as a consequence of a “strain replacement” or through coinfection with different isolates
197 being collected—completely by chance—at the different samplings (31). In addition, the
198 BOX-PCR diversity among the isolates recovered throughout the course of prolonged
199 infection demonstrated that those clones isolated during the first year or years of infection
200 belonged to different BOX subtypes (B6–B8) with the B8 subtype being predominant—
201 that finding probably since the sample collection overlapped with the outbreak period
202 (2004–2005). In contrast, after a long-lasting infection (more than three years of being
203 colonized by *B. contaminans*), either the B1 or B8 BOX subtypes became predominant,
204 and were likely coexistent (31). Therefore, we conclude that the BOX-subtype diversity of
205 the infecting strains responsible for the chronic cases investigated seemed to decrease over
206 the time of infection, with B1 and B8 subtypes being the ones that mostly persisted—and
207 apparently replacing the initially infecting strains. This type of occurrence was found to be
208 common in *P. aeruginosa* infections; where after a period of recurrent colonization, the
209 CF patients became colonized permanently with a single lineage (17, 25). Once this *P.*
210 *aeruginosa* lineage became adapted to the hostile environment of the CF lung (*i. e.*, as the
211 “adapted dominant epidemic strain”), that bacterium could persist for several decades so
212 as to overcome both the host defense mechanisms and any intensive antibiotic therapy (14,
213 17, 26, 32).

214 In conclusion, the species *B. contaminans* was indeed able to develop chronic
215 colonization in CF patients. Especially isolates belonging to the B8 and B1 BOX subtypes
216 persisted during the 7 years of our surveillance, either infecting CF patients for the first
217 time (Fig. 3C) or colonizing CF patients chronically. We therefore can infer that the
218 aforementioned genotypes can survive in certain ecological niches or industrial products,
219 spread among patients, and most readily adapt to the lungs of CF patients. This research
220 has enabled a deeper understanding of the diversity of the *B. contaminans* isolates
221 recovered from CF patients treated in Argentina. Since a knowledge of the currently

222 infectious strains is critical in prescribing patient treatment and making prognoses, isolates
223 characterized by the B1 and B8 subtypes have now become the object of ongoing
224 investigations in our laboratory. This and the forthcoming information will provide deeper
225 insights into the strategies employed by *B. contaminans* to adapt and persist within CF
226 patients.

227

228 **ACKNOWLEDGEMENTS**

229 This work was supported by the Agencia Nacional de Promoción Científica y
230 Tecnológica (grant BID 1728/OC-AR-PICT 34836) and Proyecto Extensión Fac. Cs.
231 Exactas, UNLP. A. Bosch is member CIC PBA, and P. Martina is a CONICET fellowship.
232 We would like to thank Julio Figari for his technical assistance. We are grateful to Laura
233 Galanternik for providing the *B. cepacia* complex reference strains and to Dr. Donald F.
234 Haggerty, a native English speaker, for editing the final version of the manuscript.

235

236 **Table 1.** *Burkholderia cepacia* complex species recovered from 21 patients over the time
 237 period of their chronic infections.

238

<i>Burkholderia cepacia</i> complex species recovered from the initial sample ^a	Species recovered in subsequent samples	Number of patients
<i>B. contaminans</i>	<i>B. cenocepacia</i>	4
<i>B. cepacia</i>	<i>B. vietnamiensis</i>	2
<i>B. cenocepacia</i>	<i>B. contaminans</i> , <i>B. cenocepacia</i>	2 ^b
<i>B. contaminans</i>	<i>B. contaminans</i>	5 ^c
<i>B. contaminans</i>	<i>B. contaminans</i>	8 ^d

239

240 ^a Identification of the first available clinical isolates recovered from the chronic patients.

241 ^b Species different from the initial one was detected and then, in later cultures, the initial
 242 species was found again, indicating probable species co-infection.

243 ^c The same BOX-subtype was detected in subsequent cultures.

244 ^d Different BOX subtype patterns were detected in subsequent sputum cultures.

245

246

247 **LEGENDS TO THE FIGURES**

248

249 **Fig. 1.** Phylogenetic tree of the 79 *B. contaminans* CF isolates (Table S1 in Supplementary
 250 Material) based on the analysis of a 900 bp *recA* fragment sequence using the neighbor-
 251 joining method. Bootstrap values greater than 70% are shown for 1,500 replicates. Five *B.*
 252 *contaminans* reference sequences obtained from the GenBank sequence database were
 253 included in the analysis (ascension numbers indicated in brackets). *RecA*-gene-sequence
 254 types (*recA*-STs) were obtained by comparing the sequence of a 398 bp *recA* fragment
 255 against the public MLST database (<http://pubmlst.org/bcc/>). *B. xenovorans* was used as
 256 the outgroup.

257

258

259 **Fig. 2.** Dendrogram showing the relatedness among representative BOX-PCR
 260 fingerprinting patterns obtained from *Burkholderia contaminans* *recA*-ST-71 clinical
 261 isolates. The dendrogram was produced by the UPGMA method. The scale bars indicate
 262 the percent similarity. Clusters were delineated with an 85%-similarity cut-off value as
 263 indicated by the heavy vertical line. One isolate representative of each pattern is indicated.
 264 For the key to the abbreviations used for the medical centers, *cf.* the text.

265

266 **Fig. 3.** Distribution of BOX-PCR subtypes (B1–B10) of *B. contaminans* isolates with the
 267 *recA*-ST-71 allele obtained over a 7-year period with only the first isolate recovered from
 268 each patient being considered: A) Incidence; B) Geographical distribution; C) Temporal
 269 distribution.

270

271

References

1. Assaad W., M. Magalhães, M. Plesa, C. A. Hart, P. Cornelis, and C. Winstanley. 2006. Identical *Burkholderia cepacia* complex strain types isolated from multiple patients attending a hospital in Brazil. *J. Med. Microbiol.* **55**:247-9.
2. Baldwin A., E. Mahenthiralingam, K. M. Thickett, D. Honeybourne, M. C. J. Maiden, J. R. Govan, D. P. Speert, J. J. LiPuma, P. Vandamme, and C. G. Dowson. 2005. Multilocus sequence typing scheme that provides both species and strain differentiation for the *Burkholderia cepacia* complex. *J. Clin. Microbiol.* **43**:4665.
3. Bernhardt S., T. Spilker, T. Coffey, and J. J. LiPuma. 2003. *Burkholderia cepacia* complex in cystic fibrosis: frequency of strain replacement during chronic infection. *Clin. Infect. Dis.* **37**:780-5.
4. Biddick R., T. Spilker, A. Martin, and J. J. LiPuma. 2003. Evidence of transmission of *Burkholderia cepacia*, *Burkholderia multivorans* and *Burkholderia dolosa* among persons with cystic fibrosis. *FEMS Microbiol. Lett.* **228**:57-62.
5. Bosch A., A. Miñán, C. Vescina, J. Degrossi, B. Gatti, P. Montanaro, M. Messina, M. Franco, C. Vay, J. Schmitt, D. Naumann, and O. Yantorno. 2008. Fourier transform infrared spectroscopy for rapid identification of nonfermenting gram-negative bacteria isolated from sputum samples from cystic fibrosis patients. *J. Clin. Microbiol.* **46**:2535-46.
6. Campana S., G. Taccetti, N. Ravenni, F. Favari, L. Cariani, A. Sciacca, D. Savoia, A. Collura, E. Fiscarelli, G. D. Intinis, M. Buseti, A. Cipolloni, A. Aprile, E. Provenzano, I. Collebrusco, P. Frontini, G. Stassi, M. Trancassini, D. Tovagliari, A. Lavitola, C. J. Doherty, T. Coenye, J. R. W. Govan, and P. Vandamme. 2005. Transmission of *Burkholderia cepacia* Complex: Evidence for New Epidemic Clones Infecting Cystic Fibrosis Patients in Italy. *J. Clin. Microbiol.* **43**:5136-5142.
7. Chen J. S., K. a Witzmann, T. Spilker, R. J. Fink, and J. J. LiPuma. 2001. Endemicity and inter-city spread of *Burkholderia cepacia* genomovar III in cystic fibrosis. *J. Pediatr.* **139**:643-9.
8. Coenye T., T. Spilker, A. Martin, and J. J. LiPuma. 2002. Comparative Assessment of Genotyping Methods for Epidemiologic Study of *Burkholderia cepacia* Genomovar III. *J. Clin. Microbiol.* **40**:3300-3307.
9. Coenye T., P. Vandamme, J. R. W. Govan, and J. J. LiPuma. 2001. MINIREVIEW Taxonomy and Identification of the *Burkholderia cepacia* Complex. *J Clin. Microbiol.* **39**:3427-3436.
10. Currie B. J., D. Gal, M. Mayo, L. Ward, D. Godoy, B. G. Spratt, and J. J. LiPuma. 2007. Using BOX-PCR to exclude a clonal outbreak of melioidosis. *BMC Infect. Dis.* **7**:68.

- 322 11. Dalmastri C., A. Fiore, C. Alisi, A. Bevivino, S. Tabacchioni, G. Giuliano, A.
323 R. Sprocati, L. Segre, E. Mahenthiralingam, L. Chiarini, and P. Vandamme.
324 2003. A rhizospheric *Burkholderia cepacia* complex population: genotypic and
325 phenotypic diversity of *Burkholderia cenocepacia* and *Burkholderia ambifaria*.
326 FEMS Microbiol. Ecol. **46**:179-87.
327
- 328 12. Detsika M. G., J. E. Corkill, M. Magalha, K. J. Glendinning, C. A. Hart, and
329 C. Winstanley. 2003. Molecular Typing of, and Distribution of Genetic Markers
330 among, *Burkholderia cepacia* Complex Isolates from Brazil. J. Clin.
331 Microbiol. **41**:4148-4153.
332
- 333 13. Drevinek P., and E. Mahenthiralingam. 2010. *Burkholderia cenocepacia* in
334 cystic fibrosis: epidemiology and molecular mechanisms of virulence. Eur. Soc.
335 Clin. Microbiol. Infect. Dis **16**:821-30.
336
- 337 14. Feliziani S., A. M. Luján, A. J. Moyano, C. Sola, J. L. Bocco, P. Montanaro, L.
338 F. Canigia, C. E. Argaraña, and A. M. Smania. 2010. Mucoidy, quorum
339 sensing, mismatch repair and antibiotic resistance in *Pseudomonas aeruginosa*
340 from cystic fibrosis chronic airways infections. PloS One **5**:1-12.
341
- 342 15. Hauser A. R., M. Jain, M. Bar-Meir, and S. a McColley. 2011. Clinical
343 significance of microbial infection and adaptation in cystic fibrosis. Clin.
344 Microbiol. Rev. **24**:29-70.
345
- 346 16. Henry D. A., E. Mahenthiralingam, P. Vandamme, T. Coenye, and Speert
347 D.P. 2001. Phenotypic Methods for Determining Genomovar Status of the
348 *Burkholderia cepacia* Complex. J. Clin. Microbiol. **39**:1073-1078.
349
- 350 17. Jelsbak L., H. K. Johansen, A.-L. Frost, R. Thøgersen, L. E. Thomsen, O.
351 Ciofu, L. Yang, J. a J. Haagenen, N. Høiby, and S. Molin. 2007. Molecular
352 epidemiology and dynamics of *Pseudomonas aeruginosa* populations in lungs of
353 cystic fibrosis patients. Infect. Immun. **75**:2214-24.
354
- 355 18. Jordá-Vargas L., J. Degrossi, N. C. Castañeda, M. D'Aquino, M. a Valvano,
356 A. Procopio, L. Galanternik, and D. Centrón. 2008. Prevalence of indeterminate
357 genetic species of *Burkholderia cepacia* complex in a cystic fibrosis center in
358 Argentina. J. Clin. Microbiol. **46**:1151-2.
359
- 360 19. LiPuma J. J. 2010. The changing microbial epidemiology in cystic fibrosis. Clin.
361 Microbiol. Rev. **23**:299-323.
362
- 363 20. Mahenthiralingam E., P. Vandamme, M. E. Campbell, D. a Henry, a M.
364 Gravelle, L. T. Wong, a G. Davidson, P. G. Wilcox, B. Nakielna, and D. P.
365 Speert. 2001. Infection with *Burkholderia cepacia* complex genomovars in
366 patients with cystic fibrosis: virulent transmissible strains of genomovar III can
367 replace *Burkholderia multivorans*. Clin. Infect. Dis. **33**:1469-75.
368
- 369 21. Mahenthiralingam E., J. Bischof, S. K. Byrne, C. Radomski, J. E. Davies, and
370 Y. Av-gay. 2000. DNA-Based Diagnostic Approaches for Identification of
371 *Burkholderia cepacia* Complex, *Burkholderia vietnamiensis*, *Burkholderia cepacia*
372 Genomovars I and III. J. Clin. Microbiol. **38**:3165-3173.

22. Miñán A., A. Bosch, P. Lasch, M. Stämmler, D. O. Serra, J. Degrossi, B. Gatti, C. Vay, M. D'aquino, O. Yantorno, and D. Naumann. 2009. Rapid identification of *Burkholderia cepacia* complex species including strains of the novel Taxon K, recovered from cystic fibrosis patients by intact cell MALDI-ToF mass spectrometry. *Analyst* **134**:1138-1148.
23. NCCLS. 1999. Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals. Approved standard M31-A. Wayne, PA.
24. Nørskov-lauritsen N., H. K. Johansen, G. Mette, X. C. Nielsen, T. Pressler, V. Hanne, N. Høiby, M. G. Fenger, and H. V. Olesen 2010. Unusual Distribution of *Burkholderia cepacia* Complex Species in Danish Cystic Fibrosis Clinics May Stem from Restricted Transmission between Patients. *J Clin Microbiol.* **48**: 2981-3.
25. Rau M. H., S. K. Hansen, H. K. Johansen, L. E. Thomsen, C. T. Workman, K. F. Nielsen, L. Jelsbak, N. Høiby, L. Yang, and S. Molin. 2010. Early adaptive developments of *Pseudomonas aeruginosa* after the transition from life in the environment to persistent.
26. Smith E. E., D. G. Buckley, Z. Wu, C. Saenphimmachak, L. R. Hoffman, D. a D'Argenio, S. I. Miller, B. W. Ramsey, D. P. Speert, S. M. Moskowitz, J. L. Burns, R. Kaul, and M. V. Olson. 2006. Genetic adaptation by *Pseudomonas aeruginosa* to the airways of cystic fibrosis patients. *Proc. Natl. Acad. Sci. USA* **103**:8487-92.
27. Vanlaere E., A. Baldwin, D. Gevers, D. Henry, E. De Brandt, J. J. LiPuma, E. Mahenthiralingam, D. P. Speert, C. Dowson, and P. Vandamme. 2009. Taxon K, a complex within the *Burkholderia cepacia* complex, comprises at least two novel species, *Burkholderia contaminans* sp. nov. and *Burkholderia lata* sp. nov. *Int. J. Syst. Evol. Microbiol* **59**:102-11.
28. Vanlaere E., J. J. LiPuma, A. Baldwin, D. Henry, E. De Brandt, E. Mahenthiralingam, D. Speert, C. Dowson, and P. Vandamme. 2008. *Burkholderia latens* sp. nov., *Burkholderia diffusa* sp. nov., *Burkholderia arboris* sp. nov., *Burkholderia seminalis* sp. nov. and *Burkholderia metallica* sp. nov., novel species within the *Burkholderia cepacia* complex. *Int. J. Syst. Evol. Microbiol.* **58**: 1580–1590.
29. Vermis K., T. Coenye, E. Mahenthiralingam, H. J. Nelis, and P. Vandamme. 2002. Evaluation of species-specific recA-based PCR tests for genomovar level identification within the *Burkholderia cepacia* complex. *J. Med. Microbiol.* **51**:937-40.
30. Versalovic J., T. Koeuth, and J. R. Lupski. 1991. Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. *Nucl. Acids Research* **19**:6823-31.59.

- 422 31. **Yang J. H., T. Spilker, and J. J. LiPuma.** 2006. Simultaneous coinfection by
423 multiple strains during *Burkholderia cepacia* complex infection in cystic fibrosis.
424 *Diag. Microbiol. Infect. Dis.* **54**:95-8.
425
- 426 32. **Yang L., L. Jelsbak, R. L. Marvig, S. Damkiær, C. T. Workman, M. H. Rau,**
427 **S. K. Hansen, A. Folkesson, H. K. Johansen, O. Ciofu, N. Høiby, M. O.**
428 **Sommer, and S. Molin.** 2011. Evolutionary dynamics of bacteria in a human host
429 environment. *Proc. Natl. Acad. Sci. USA* **108**:7481-6.
430
- 431 33. **Zhou J., E. Garber, M. Desai, and L. Saiman.** 2006. Compliance of clinical
432 microbiology laboratories in the United States with current recommendations for
433 processing respiratory tract specimens from patients with cystic fibrosis. *J. Clin.*
434 *Microbiol.* **44**:1547–1549.
435
436
437
438
439
440
441

Fig. 1





